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QTL analysis for rice grain length and fine mapping of an identified QTL with stable and major effects

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Abstract Grain length in rice plays an important role in determining rice appearance, milling, cooking and eating quality. In this study, the genetic basis of grain length was dissected into six main-effect quantitative trait loci (QTLs) and twelve pairs of epistatic QTLs. The stability of these QTLs was evaluated in four environments using an F_7 recombinant inbred line (RIL) population derived from the cross between a *Japonica* variety, Asominori, and an *Indica* variety, IR24. Moreover, chromosome segment substitution lines (CSSLs) harboring each of the six main-effect QTLs were used to evaluate gene action of QTLs across eight environments. A major QTL denoted as *qGL-3a*, was found to express stably not only in the isogenic background of Asominori but also in the recombinant background of Asominori and IR24 under multiple environments. The IR24 allele at *qGL-3a* has a positive effect on grain length. Based on the test of advanced backcross progenies, *qGL-3a* was dissected as a single Mendelian factor, i.e., long rice grain was controlled by a recessive gene *gl-3*. High-resolution genetic and physical maps were further constructed for fine mapping *gl-3* by using 11 simple sequence repeat (SSR) markers designed using sequence information from seven BAC/PAC clones and a BC_4F_2 population consisting of 2,068 individuals. Consequently, the *gl-3* gene was narrowed down to a candidate genomic region of 87.5 kb long defined by SSR markers RMw357 and RMw353 on chromosome 3, which provides a basis for map-based cloning of this gene and for marker-aided QTL pyramiding in rice quality breeding.

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Introduction

Grain length and shape determine appearance in rice, and affect milling, cooking and eating quality, and are therefore important agronomic traits in rice breeding (Luo et al. 2004). Preferences for grain shape vary across different consumers. Long and slender grain varieties are preferred in most Asian countries including China, India, Pakistan and Thailand, and also in the USA, while short grain cultivars are preferred in Japan and Sri Lanka. In addition, grain dimension is an important indicator of the evolution of cereal crops because humans tended to select large seeds during the early domestication, as evidenced by the fact that most cultivated species have larger seeds than their wild relatives (Harlan 1992). However, small seed is usually favored by natural selection because it is frequently associated with more seeds per plant, early maturity, and wider geographic distribution. Therefore, from the standpoints of both biological development and breeding, it is necessary to understand the genetic basis and formation mechanism of rice grain shape.

Since rice grain length is quantitatively inherited (McKenzie and Rutger 1983), it is difficult for breeders to efficiently improve grain appearance using conventional selection methods. Thus, it should be particularly helpful for enhancing breeding efficiency to use markers closely linked to genes or major quantitative trait loci (QTLs) for grain length in order to screen target genotypes directly in early generations. Independent studies have identified many QTLs associated with rice grain length using primary mapping populations (Huang et al. 1997; Redoña and Mackill 1998; Tan et al. 2000; Li et al. 2004b; Aluko et al. 2004). However, in all these studies the grain length was evaluated in a single environment, so the stability of the resultant QTLs could not be

determined. The expression stability of a QTL is critical in determining its usefulness in marker-assisted selection (MAS) breeding. Of the 21 QTLs for grain length detected in these studies cited above, one has been consistently identified near the centromeric region of rice chromosome 3, with the average percentage of phenotypic variation explained (PVE) of 33.5%. One basic question regarding this QTL still remains, i.e., whether the QTL corresponds to only one locus or to a chromosomal region consisting of a cluster of genes, each with relatively small genetic effects. This question has to be answered before we further develop MAS strategies or clone this QTL. Therefore, it is necessary to establish whether the QTL is a single Mendelian factor and to narrow down its location on the genetic linkage map.

Primary populations such as $F_{2:3}$, RIL, BC and DH are not appropriate for fine mapping of a single QTL, because they segregate whole parental chromosomal segments simultaneously (Yamamoto et al. 2000). On the contrary, chromosome segment substitution lines (CSSLs) or near-isogenic lines (NILs) have distinct advantages for QTL identification. Most importantly, genetic interactions between donor alleles are limited to those between genes on homozygous substituted tracts since each CSSL/NIL carries one or a few donor segments in the near-isogenic background of the recurrent parent, thus reducing the effects of interferences from genetic background (Howell et al. 1996). In addition, secondary F_2 and F_3 populations can be derived from a further backcross between a selected CSSL/NIL and the recurrent parent, and then be used in the fine mapping and positional cloning of a QTL (Yamamoto et al. 1998; Frary et al. 2000; Yano et al. 2000; Yan et al. 2004). Recently, a grain-weight QTL, *gw*3.1, was narrowed down to a 93.8-kb interval in the centromeric region of rice chromosome 3, by using a set of NILs derived from the cross between an *Oryza sativa*, cv, Jefferson and *O. rufipogon* (IRGC105491) based on five generations of backcrossing and seven generations of selfing. The dominant *O. rufipogon* allele at *gw*3.1 conferred small seed size and might associate with domestication in rice (Li et al. 2004a). Otherwise, stability of QTLs for rice grain dimension was studied across eight environments using a population of 66 CSSLs derived from three backcrosses of IR24 to Asominori (Asominori/IR24//3*Asominori) (Wan et al. 2005). A grain-length QTL, *qGL-3*, with an average PVE of 32.8%, was mapped to an 18.1-cM interval in the centromeric region of rice chromosome 3 and was consistently detected in all the eight environments, indicating that the IR24 allele at *qGL-3* had a significant and stable effect on increasing grain length in the homozygous genetic background of Asominori.

The objectives of this study were: (1) to determine whether or not the epistatic effect of QTLs in the recombinant background of Asominori and IR24 has a significant influence on expression stability of the IR24 allele at *qGL-3a*; (2) to establish whether the stable major QTL *qGL-3a* is a single Mendelian factor, *gl-3* gene, by using secondary F_2 and F_3 populations derived from a cross between the target CSSL and the recurrent parent; and (3) to locate the *gl-3* gene to a narrow genomic region based on SSR markers produced from sequence information of the *japonica* cultivar, cv. Nipponbare, and then compare the location relationship between the *gw*3.1 (Li et al. 2004a) and *gl-3* genes from different rice germplasm, respectively.

Materials and methods

Plant materials

Four populations, RILs, CSSLs, BC_4F_2 and BC_4F_3, were used in this study.

Seventy-one F_7 RILs were derived from the cross between Asominori and IR24 by single-seed descent (Tsunematsu et al. 1996). To produce a series of CSSLs in a largely Asominori background, 19 selected RILs were crossed and then backcrossed with Asominori, without selection, until the BC_3F_1 generation. Then 66 individuals were selected at BC_3F_1 on the basis of a whole genome survey (116 RFLP loci) and they were denoted as CSSL1-CSSL66, which have representation of the whole IR24 genome, except for the 9.8 cM region defined by the interval C1468-G1015 on chromosome 3 (Kubo et al. 1999; Wan et al. 2004a).

Of four long-grain lines (CSSL16, 17, 18, and 46) harboring the *gl-3* allele, CSSL18 was used to build the secondary F_2 (BC_4F_2) and F_3 (BC_4F_3) populations by backcrossing to Asominori with subsequent self-pollination.

Field experimental design

This study was carried out in ten environments, E1-E10, including four locations and four cropping seasons (Table 1). Asominori, IR24 and their 71 RILs were grown in four environments (E1–E4), and the parental varieties and their 66 CSSLs in eight environments (E1–E8). Each experimental plot consisted of two replicates, consisting of ten rows each of ten plants, grown in a randomized block design. At maturity, each plot was harvested in bulk. After drying, grains were stored at room temperature for 3 months, and then milled using the method described by Yamamoto et al. (1995). The milled rice thus obtained was used for determining grain length.

Using a spacing pattern of 25 cm (between rows) × 13.3 cm (within rows), 214 BC_4F_2 plants were grown in environment E9, and 214 BC_4F_3 families (16 plants per family) in E10. Meanwhile, 2,068 BC_4F_2 individuals were planted in E10, of which 499 homozygous plants with long rice grain were used to precisely map the *gl-3* gene. At maturity, seeds collected from primary panicles were dried for 72 h at 50°C. Paddy rice and brown rice

Table 1 Characteristics of ten environments where the RILs, CSSLs, BC_4F_2 and BC_4F_3 populations were grown

Code	Population	Replication	Cropping location	Cropping season	Average daily air temperature (°C)	Photoperiod (sunlight/daytime, hour)	Rainfall (mm/month)
E1	RILs, CSSLs	2	Nanjing, China, N 31.2°, E 118.4°	May–Oct, 2002	23.8	6.1/13.0	129.4
E2	RILs, CSSLs	2	Jinhu, Jiangsu, China, N 32.7° , E 119.6°	May–Oct, 2002	22.6	6.7/13.2	111.5
E3	RILs, CSSLs	2	Donghai, Jiangsu, China, N 35.1°, E 118.4°	May–Oct, 2002	22.2	7.0/13.5	104.2
E4	RILs, CSSLs	2	Lingshui, Hainan, China, N 18.2° , E 108.9°	Dec, 2002–May, 2003	24.6	6.6/12.9	78.9
E5	CSSLs	2	Nanjing, China, N 31.2°, E 118.4°	May–Oct, 2001	24.3	5.8/13.0	62.1
E6	CSSLs	2	Jinhu, Jiangsu, China, N 32.7° , E 119.6°	May–Oct, 2001	23.3	6.9/13.1	74.1
E7	CSSLs	2	Donghai, Jiangsu, China, N 35.1°, E 118.4°	May–Oct, 2001	22.9	7.0/13.4	121.8
E8	CSSLs	2	Lingshui, Hainan, China, N 18.2° , E 108.9°	Dec, 2001–May, 2002	25.4	6.7/13.0	42.2
E9	BC_4F_2	1	Nanjing, China, N 31.2°, E 118.4°	May–Oct, 2003	24.9	6.3/13.1	142.6
E10	BC_4F_2, BC_4F_3	1	Nanjing, China, N 31.2°, E 118.4°	May–Oct, 2004	23.6	6.0/13.0	93.2

were used to evaluate grain length, grain width and length-width ratio (LWR).

Phenotypic evaluation

The lengths of twenty milled rice grains randomly selected from each line (Asominori, IR24, RIL or CSSL) were estimated as the lengthwise distance between opposite tips using an electronic digital caliper (Guanglu Measuring Instrument Co. Ltd, China) with a precision of 0.1 mm.

Using the same method, The lengths were evaluated for twenty paddy rice and brown rice samples from Asominori, CSSL18 and each plant in the BC_4F_2 population and BC_4F_3 families. Meanwhile, the widths of twenty paddy rice and brown rice samples were estimated as the largest transverse distance of 20 grains using the electronic digital caliper, respectively. The average length and width of 20 grains were used as the phenotypic value of grain length and grain width, respectively. The LWR, which represents grain shape, was given by the ratio grain length/grain width.

DNA preparation and PCR protocol

DNA was extracted from fresh leaves of BC_4F_2 individuals using the method described by Dellaporta et al. (1983). The extracted DNA was dissolved in TE buffer (10 mM Tris, 0.1 mM EDTA) and tested for quality and quantity using a MBA 2000 UV/VIS Spectrometer (Perkin Elmer Co.). Suitable DNA samples were diluted to 20 ng/μl with double distilled water and stored at 4°C for polymerase chain reaction (PCR).

PCR was performed using the procedure of Chen et al. (1997) with minor modifications. Briefly, a total volume of 10 μl contained 10 ng template DNA, 0.2 μM of each primer, 50 μM of dNTPs, 0.5 unit of *Taq* polymerase, and 1 μl of 10× buffer with 1.5 mM $MgCl_2$. Thirty-five cycles were carried out, with an initial 5-min period at 94°C followed by cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C, and a final 7-min period at 72°C. PCR products were separated on an 8% non-denaturing polyacrylamide gel and detected using the silver staining method of Sanguinetti et al. (1994).

SSR marker analysis

The required density of markers in the centromeric region harboring the *gl-3* gene was achieved by using previously published SSR and EST markers on rice chromosome 3 (McCouch et al. 2002; Wu et al. 2002) as well as SSR markers developed in this study. New SSR markers were produced from the publicly available rice genome sequence of Nipponbare (sequenced by the International Rice Genome Sequencing Project, http://www.rgp.dna.affrc.go.jp), by using the Primer 5 and SSRIT procedures (http://www.gramene.org/db/searches/ssrtool). The polymorphism between the parents was predicted by comparing sequences from Nipponbare and the *indica* cultivar, 93–11 (sequenced by the Beijing Genomics Institute, http://www.rice.genomics.org.cn/index.jsp). Primer sequences, map position and amplified length of 16 new SSR markers used in this study are listed in Table 2.

Data analysis

QTL analysis

The linkage map of the Asominori/IR24 RIL population constructed by Tsunematsu et al. (1996), including 375 RFLP markers, was used in this study for QTL analysis. Tests of QTL main effects, epistatic interactions and QTL × environment interactions (QEI) were carried out using the computer program QTL Mapper 1.0 (Wang et al. 1999). QTL main effects were estimated using the maximum-likelihood estimation method, while QEI effects were predicted by the BLUP method. In the mixed linear model, the environmental effect was regarded as random. Thus, the significance test for the predicted QEI effects had very low power. As a remedy, the Bayesian test was used for the estimation of QTL main effects and QEI effects, and also for the significance test. In this study, the LR value corresponding to $P = 0.005$ (equivalent to LOD = 4.03) was used as the threshold for claiming the presence of main-effect or epistatic QTLs. The relative contribution was calculated as the PVE by the QTL.

Gene action of main-effect QTLs was evaluated by a *t*-test to show the presence of significant differences between the phenotypic values of the recurrent parent Asominori and those of CSSLs harboring the QTL allele derived from the donor parent IR24 (Wan et al. 2004a).

Primary mapping of the gl-3 gene

A total of 214 BC_4F_2 plants were genotyped using 11 SSR and 1 EST markers to construct a small-scale linkage map. Among these molecular markers, five were new SSRs developed in our laboratory. Phenotypic performance of the 214 BC_4F_2 plants was mearured and then classified into two types: long rice grain such as IR24 or CSSL18, and short rice grain such as Asominori. Based on the segregation of grain length in the 214 BC_4F_3 families, the BC_4F_2 population was further partitioned into three groups, long-grain plants (genotype *gl-3gl-3*), segregating short-grain plants (genotype *Gl-3gl-3*), and non-segregating short-grain individuals (genotype *Gl-3Gl-3*). Small-scale genetic mapping of the *gl-3* gene was performed using the Mapmaker/Exp 3.0 program to combine the genotypic data of molecular markers and the *gl-3* gene (Lander et al. 1987).

High-resolution mapping

For high-resolution mapping of the *gl-3* gene, the bulked-extreme and recessive-class approach as described by Zhang et al. (1994) was used for calculating the recombination frequencies (c) between the *gl-3* gene and 11 newly developed SSR markers in the 499 homozygous BC_4F_2 plants with long grain, out of 2,068 individuals. Thus $c = (N_1 + N_2/2)/N$, where N is the total number of long-grain plants surveyed, N_1 is the number of long-grain individuals with the banding pattern of the short-grain parent, and N_2 is the number of long-grain plants with heterozygous banding patterns.

Results

Phenotypic variation of grain length in the RIL population

The distributions of grain length in the RIL population grown in four environments (E1–E4) are shown in Fig. 1. A significant difference was observed for grain length of milled rice between the two parents, Asominori

Table 2 Sixteen SSR markers designed by using sequence information of Nipponbare in the centromeric region of rice chromosome 3

Marker name	Motif length	BAC location	[a] Size (bp)	Forward primer (5′–3′)	Reverse primer (5′–3′)	[b] AT (°C)
RMw305	$(CT)_9$	OSJNBb0004M10	206	ATGAAGTCTAAAGCCCACC	CTATACCGCCAACATCTAC	50
RMw306	$(AAG)_{10}$	OSJNBb0004M10	119	GATTCCAAACGTGTCCACCA	TCTTCAGACCGGGGATGAGC	60
RMw309	$(CA)_{10}$	OSJNBa0087G11	143	GCTGCTGCCTTTCACTTCA	CGCTTCCTGCTACTTCTCG	56
RMw310	$(GA)_{11}$	OSJNBa0087G11	189	TCGAAAGGGAAAGCTAATG	CTGCAAAGATCAAAGCAAA	53
RMw312	$(GA)_{16}$	OSJNBa0087G11	175	CTAACCTTCCCTAAACCTAC	TTTGTTCACTTCCTGCTACT	50
RMw314	$(TC)_{11}$	OSJNBa0015K03	160	AAAGAAAGAAAGCACCAC	GAAAGGGAAAGCTAATGT	50
RMw315	$(GT)_{10}$	OSJNBa0015K03	144	CGCTTCCTGCTACTTCTCG	TGCTGCTGCCTTTCACTTC	56
RMw319	$(TC)_{15}$	OSJNBb0041J20	163	TCGTCTCCATCAAGTTTTC	TGTTATTTGGACATGCTGA	50
RMw357	$(AT)_{24}$	OSJNBb0074M06	238	CGAAATCTTAAACTAACAAC	TAAACCGCACATTCAACAC	52
RMw353	$(AG)_{16}$	OSJNBa0002D18	202	TGGAGCTGTGGACTACTGGA	TCCCTGAGCCTACCTGTCAT	55
RMw323	$(TA)_{12}$	OSJNBa0030J19	221	ATACACCTCAAGTTTACCCAA	TCCTCCTCCCCTTCTTC	51
RMw324	$(AG)_{16}$	OSJNBa0030J19	170	CTTGGCGTCAATCTGTT	TTATCCCTGAGCCTACCT	49
RMw327	$(AG)_{17}$	OSJNBb0059G13	170	ACCTTTGTATCTGTGCGAGAA	CCGTTGCAGCCTTTGCT	56
RMw329	$(TA)_{26}$	OSJNBb0059G13	207	TGGATTAGCTCCCTCAATT	TCCCTCTACGCCTAAAGAT	52
RMw330	$(GCC)_8$	OSJNBa0032H19	234	TGGTCGGTTGCTTACCTTGC	CCCGCCGCTGTATAAATCC	60
RMw331	$(AT)_{25}$	OSJNBa0032H19	166	CATGGCCCAATAAGGATC	AGGGAGGACAATTAGACGT	52

[a]Size means the size of PCR product in Nipponbare
[b]AT represents annealing temperature (°C)

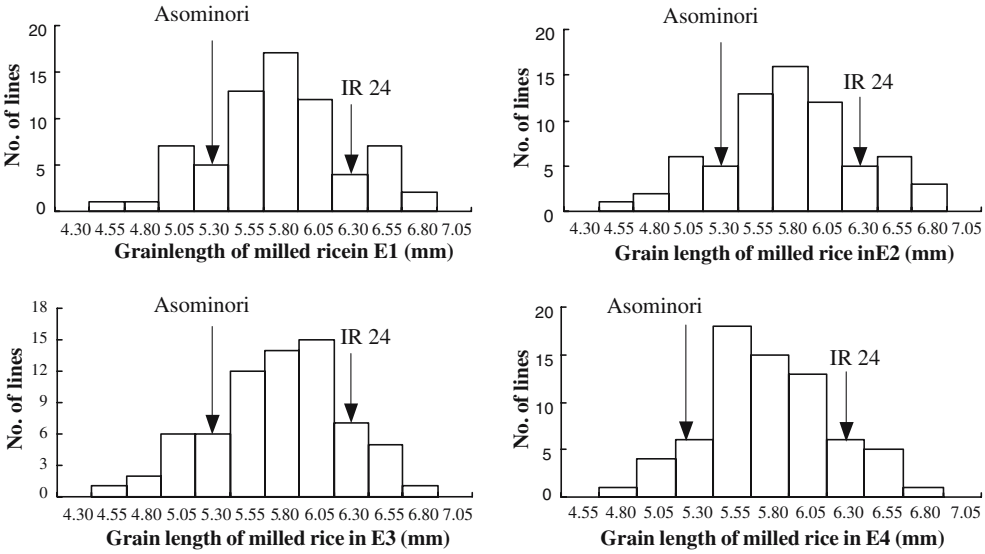


Fig. 1 Phenotypic distribution of grain length of milled rice in the Asominori/IR24 RIL population across four environments

and IR24. Grain length showed a pattern of continuous and approximately normal distributions across all the four environments, indicating quantitative inheritance of the character studied. Meanwhile, transgressive segregation of grain length could be observed in the RIL population across the four environments. In addition, ANOVA indicated that variances among the genotypes (the RILs) were highly significant for grain length, but those among the four environments were not significant. The genotype × environment interactions were also highly significant, but contributed only about 1% of the total phenotypic variation in grain length.

Grain-length main-effect QTLs and their interactions with environments in the RIL population

Six main-effect QTLs for grain length were identified in four environments and mapped to five rice chromosomes with LOD values between 7.30 and 28.94 (Table 3). Among them, *qGL-3a* was consistently detected in these four environments and mapped to the interval C80–C1677 in the centromeric region of chromosome 3 (Harushima et al. 1998), with an average PVE of 34.6%. The IR24 allele at *qGL-3a* increased grain length by an average of 0.28 mm across the four environments. Moreover, the expression stability of *qGL-3a* was relatively high since this QTL showed non-significant interactions with E1, E2, E3 and E4. QTL *qGL-2* was mapped to the interval C777-R1989 on chromosome 2 in E1, E2 and E3, and on average accounted for 8.0% of phenotypic variation, with the IR24 allele providing a positive effect of 0.13 mm. Four additional QTLs (*qGL-3b, qGL-5*, *qGL-7* and *qGL-9*) were identified in only one environment, with PVEs from 10.3 to 19.1% and showing significant QEI effects.

Grain-length epistatic QTLs and their interactions with environments in the RIL population

Twelve pairs of epistatic QTLs for grain length distributed on ten chromosomes were identified in four environments, with an average LOD value of 9.78 and

Table 3 The putative main-effect QTLs and their environmental interactions for grain length of milled rice detected using the Asominori/IR24 RIL population

Locus	Chr.	Marker interval	Environment	LOD score	[a] PVE (%)	Additive effect (mm)	Positive allele	QEI effect
qGL-3a	3	C80-C1677	E1	27.79	32.2	−0.26	[b] I	[c] NS
			E2	26.92	41.3	−0.26	I	NS
			E3	28.94	35.6	−0.31	I	NS
			E4	15.28	29.4	−0.27	I	NS
qGL-2	2	C777-R1989	E1	7.73	5.8	−0.11	I	NS
			E2	7.60	7.4	−0.11	I	NS
			E3	11.98	10.7	−0.17	I	−0.06**
qGL-3b	3	XNpb249-C1468	E4	7.30	11.6	0.17	A	−0.06*
qGL-5	5	Y1060L-R569	E4	7.85	10.3	−0.16	I	−0.10**
qGL-7	7	XNpb379-XNpb268	E1	15.97	19.1	−0.20	I	−0.12**
qGL-9	9	XNpb339-C796C	E3	12.16	10.7	0.17	A	0.08**

[a]PVE represents the percentage of phenotypic variation explained
[b]A and I represent the positive effects of QTL contributed by Asominori and IR24 alleles, respectively
[c]NS means the non-significant effects of the QEI

PVE of 5.2% (Table 4). Of these, one epistatic QTL pair was detected in all the four environments, two pairs in two of the environments, and nine pairs in only one environment. Five digenic interactions occurred between a main-effect QTL and a modifying factor. One interaction occurred between two loci on chromosome 2, while the eleven other epistatic QTL pairs involved loci on separate chromosomes. Significant epistatic QTL × environment (E-AA_{ij}) effects were identified for eight of the 12 epistatic QTL pairs and these E-AA_{ij} effects differed greatly in both direction and magnitude under different environments.

Gene action of main-effect QTLs in the CSSL population

In order to confirm gene action of the six main-effect QTLs identified in this study, we selected 17 CSSLs from the population of 66 lines (Fig. 2) (Kubo et al. 1999). In CSSL16, 17, 18 and 46, the IR24 chromosomal segment harboring *qGL-3a* (defined by RFLP markers R3156 and C1677) was substituted in the genetic background of Asominori. The IR24 segment in the interval G1340-XNpb132 including *qGL-2* was substituted in CSSL7, 10, 19, 38 and 39. Similarly, the three IR24 segments defined by R3166 and R569, R1789 and C924, and XNpb13 and C609, harboring *qGL-5*, *qGL-7* and *qGL-9*, were substituted in three (CSSL28, 29 and 32), two (CSSL46, 47), and four (CSSL50, 52, 54 and 55) CSSLs, respectively. In addition, gene action of *qGL-3b* could not be verified because CSSLs in the interval C1468-G1015 as described above were not available.

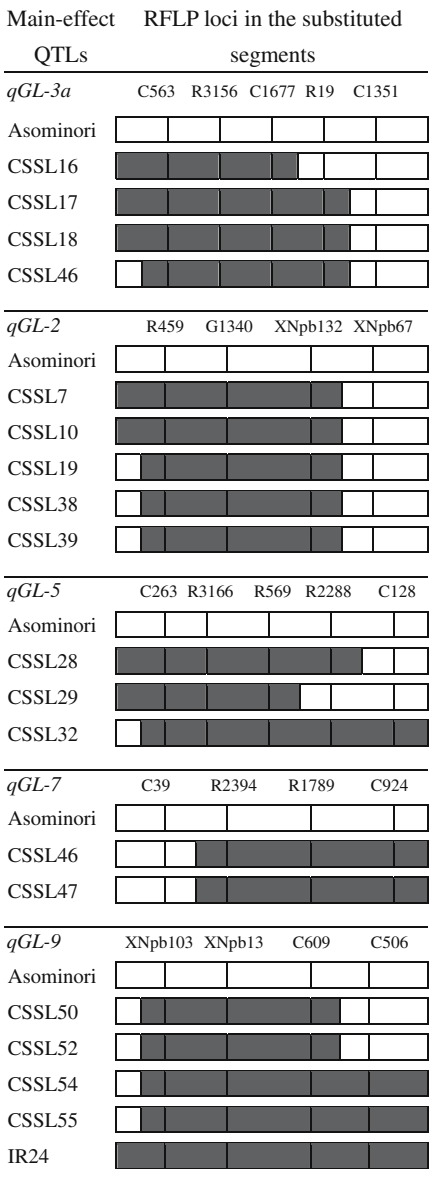
t-Tests demonstrated significant differences between grain length of milled rice of Asominori and that of each of four CSSLs carrying *qGL-3a* (Fig. 2), indicating that gene action of *qGL-3a* was significant and stable across all the eight environments. Most interestingly, the IR24 *qGL-3a* allele increased grain length by an average of 0.28 mm in the RIL population (Table 3), but only 0.26 mm in the CSSL population (Wan et al. 2005). The effect difference (0.02 mm) of this QTL could be explained by the digenic interaction between *qGL-3a* and a modifying factor, with an AA_{ij} effect of 0.03* (Table 4), which did not exist in the CSSL population. These results demonstrate that the IR24 *qGL-3a* allele could express stably not only in the Asominori genetic background but also in the recombinant background of Asominori and IR24 with epistatic interaction across multiple environments.

For *qGL-2*, significant differences of grain length between Asominori and five target CSSLs were observed only in from one to seven environments (Fig. 2). The effect direction of the IR24 *qGL-2* allele in CSSL10, 19, 38 and 39 was consistent with that in the RIL population, however, the reverse direction was found in CSSL7 (Fig. 2; Table 3). Otherwise, the other four segments on chromosomes 1, 8, 10 and 12 were also substituted in CSSL7, but these segments not in CSSL10, 19, 38 and 39 (Kubo et al. 1999). Therefore, gene action of *qGL-2* was sensitive to the genetic background and environmental conditions. Additionally, although the effect directions of *qGL-5*, *qGL-7* and *qGL-9* were consistent in the target CSSLs and RIL population, gene action of these three QTLs showed strong environment-specificity (Fig. 2; Table 3).

Table 4 The putative epistatic QTLs and their environmental interactions for grain length of milled rice detected using the Asominori/IR24 RIL population

Chr.	Interval *i*	[a]M-QTL	Chr.	Interval *j*	M-QTL	Environment	LOD score	[b]PVE	[c] AA*ij*	[d] E-AA*ij*
4	C335-C621B		10	C1166-C1286		E1	5.16	3.36	−0.09**	NS
						E2	12.74	3.68	−0.10**	0.022*
						E3	8.15	6.75	−0.13**	NS
						E4	8.62	12.61	−0.17**	−0.036*
4	XNpb247-R2373		5	XNpb251-XNpb105		E2	6.66	3.68	−0.10**	NS
						E3	7.28	6.75	−0.13**	−0.047**
2	C796A-XNpb132		2	XNpb223-R459		E2	6.21	1.66	0.07**	NS
						E3	11.49	11.34	0.17**	0.090**
1	C3029C-C2340		3	C80-C1677	*qGL-3a*	E2	28.09	2.35	0.03*	NS
1	G2200-C86		11	C410-C1350		E1	7.95	5.02	−0.11**	−0.045**
2	C777-R1989	*qGL-2*	4	XNpb177-C600		E2	9.99	5.90	0.04*	NS
3	C1351-R19		8	XNpb56-R2382		E1	6.14	2.03	0.07**	NS
3	XNpb249-C1468	*qGL-3b*	11	C83B-C3029A		E1	5.75	5.02	−0.11**	NS
3	C161B-XNpb392		12	C751-XNpb88		E2	11.57	8.27	0.15**	0.047**
4	Ky4-R2737		5	XNpb105-R1553		E3	7.01	4.83	0.11**	0.084**
5	R569-XNpb251	*qGL-5*	12	C718B-C104A		E2	14.12	6.21	0.13**	−0.039*
9	XNpb339-C796C	*qGL-9*	11	C1003A-C1172		E2	9.40	4.45	−0.11**	−0.048*

[a]M-QTL means main-effect QTL
[b]PVE represents the percentage of phenotypic variation explained
[c]AA*ij* means the additive by additive epistatic effect of QTLs
[d]E-AA*ij* represents the interaction effect of epistatic QTL by environment



Phenotypic values of grain length of parents and target CSSLs across the eight environments

	E1	E2	E3	E4	E5	E6	E7	E8
Asominori	5.33	5.29	5.32	5.29	5.29	5.25	5.31	5.18
CSSL16	5.80**	5.87**	5.83*	5.84**	5.83**	5.81**	5.83**	5.82**
CSSL17	5.79**	5.79**	5.83*	5.77**	5.81**	5.80**	5.83**	5.81**
CSSL18	5.63**	5.65*	5.66*	5.65**	5.65*	5.65**	5.67**	5.59**
CSSL46	5.79**	5.76**	5.75*	5.75**	5.75*	5.75*	5.73**	5.76**
qGL-2	E1	E2	E3	E4	E5	E6	E7	E8
Asominori	5.33	5.29	5.32	5.29	5.29	5.25	5.31	5.18
CSSL7	5.24^{ns}	5.31^{ns}	5.23*	5.38^{ns}	5.20^{ns}	5.23^{ns}	5.22*	5.29^{ns}
CSSL10	5.32^{ns}	5.36^{ns}	5.45^{ns}	5.37^{ns}	5.28^{ns}	5.32^{ns}	5.44*	5.31^{ns}
CSSL19	5.51*	5.57*	5.57*	5.65**	5.52 *	5.56*	5.59**	5.51^{ns}
CSSL38	5.38^{ns}	5.47^{ns}	5.44*	5.22^{ns}	5.37^{ns}	5.35^{ns}	5.38^{ns}	5.16^{ns}
CSSL39	5.46*	5.51^{ns}	5.58**	5.39^{ns}	5.38^{ns}	5.42^{ns}	5.57**	5.36*
qGL-5	E1	E2	E3	E4	E5	E6	E7	E8
Asominori	5.33	5.29	5.32	5.29	5.29	5.25	5.31	5.18
CSSL28	5.36^{ns}	5.40^{ns}	5.45*	5.49**	5.40^{ns}	5.41*	5.54^{ns}	5.37^{ns}
CSSL29	5.53^{ns}	5.48^{ns}	5.60*	5.44*	5.57*	5.43^{ns}	5.51^{ns}	5.37^{ns}
CSSL32	5.49^{ns}	5.41^{ns}	5.55*	5.35^{ns}	5.46^{ns}	5.40*	5.52*	5.30^{ns}
qGL-7	E1	E2	E3	E4	E5	E6	E7	E8
Asominori	5.33	5.29	5.32	5.29	5.29	5.25	5.31	5.18
CSSL46	5.79**	5.76**	5.75*	5.75**	5.75*	5.75*	5.73**	5.76**
CSSL47	5.24^{ns}	5.37^{ns}	5.39*	5.32^{ns}	5.25^{ns}	5.30^{ns}	5.33^{ns}	5.19^{ns}
qGL-9	E1	E2	E3	E4	E5	E6	E7	E8
Asominori	5.33	5.29	5.32	5.29	5.29	5.25	5.31	5.18
CSSL50	5.44^{ns}	5.37^{ns}	5.53^{ns}	5.38^{ns}	5.35^{ns}	5.34^{ns}	5.49^{ns}	5.24^{ns}
CSSL52	5.21^{ns}	5.20^{ns}	5.13*	5.15^{ns}	5.15^{ns}	5.14^{ns}	5.20^{ns}	5.08^{ns}
CSSL54	5.34^{ns}	5.38^{ns}	5.33^{ns}	5.25^{ns}	5.29^{ns}	5.33^{ns}	5.31^{ns}	5.18^{ns}
CSSL55	5.22^{ns}	5.32^{ns}	5.24^{ns}	5.13*	5.19^{ns}	5.18^{ns}	5.33^{ns}	5.03^{ns}
IR24	5.85**	5.95**	5.98**	5.85*	5.91**	5.91**	5.98**	5.85**

Fig. 2 Grain length of milled rice between the recurrent parent Asominori and target CSSLs carrying each of five main-effect QTLs alleles in the eight environments * and ** mean the significance levels 5 and 1%, respectively. NS represents non-significant difference. The test was conducted between Asominori and IR24, or one target CSSL

Phenotypic variation of grain length, grain width and LWR in the BC_4F_2 and BC_4F_3 populations

In environment E9, the frequency distributions of grain length of paddy rice and brown rice in 214 BC_4F_2 individuals appeared to be bimodal with the boundaries of 8.30 and 5.84 mm (Fig. 3a, b). The ratio of short-grain individuals (162) to long-grain individuals (50) corresponded to the expected 3:1 segregation for a single-locus inheritance ($\chi^2 = 0.224$, $P > 0.05$). Otherwise, in BC_4F_3 progeny testing, three different phenotypes of grain length, including long-grain lines, segregating short-grain lines, and non-segregating short-grain lines, were clearly observed in E10. Therefore, it was easy to assess the genotype at *qGL-3a* of each BC_4F_2 plant and BC_4F_3 family. The long grain lines were considered to be homozygous for "CSSL18 or IR24" alleles, segregating lines as heterozygous plants, and non-segregating short-grain as homozygous for "Asominori" alleles. The numbers of families in these three groups were 50, 102 and 62, respectively. This is consistent with the Mendelian ratio 1:2:1 for a single gene inheritance ($\chi^2 = 1.553$, $P > 0.05$). These results demonstrate that QTL *qGL-3a* could be treated as a single Mendelian factor. Thus long rice grain in the BC_4F_2 population is controlled by a recessive gene, designated as *gl-3*.

Similarly, the bimodal frequencies were observed for LWR of paddy rice and brown rice in the 214 BC_4F_2 individuals, with the boundaries of 2.80 and 2.25 mm, respectively (Fig. 3e, f). The ratio of small LWR individuals (162) to large LWR individuals (50) fitted in with the expected 3:1 segregation. Meanwhile, the family numbers of 50, 102 and 62 were detected for large-LWR lines, segregating small-LWR lines and non-segregating small-LWR lines in the BC_4F_3 population, respectively, which is in agreement with the Mendelian ratio 1:2:1.

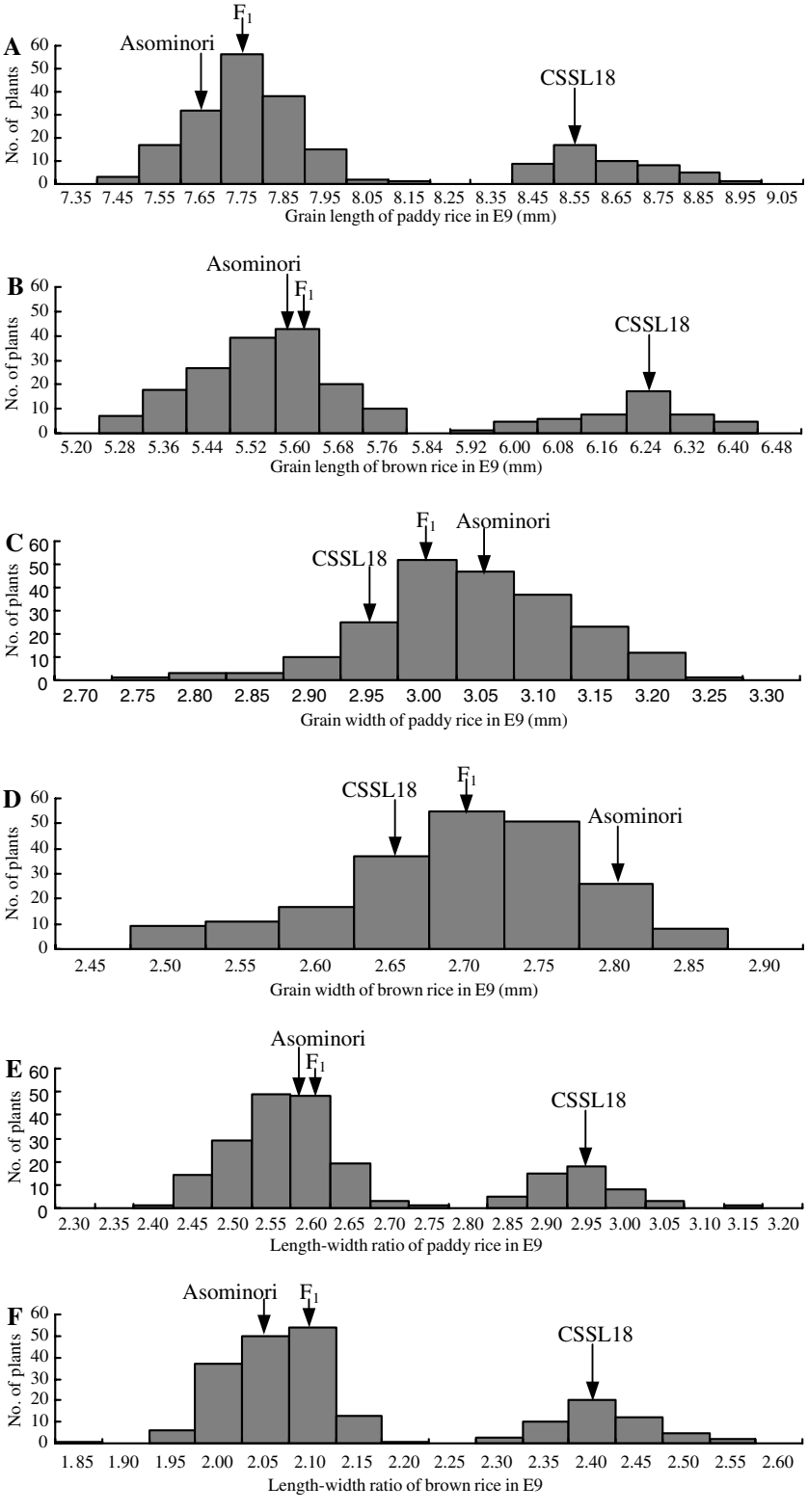


Fig. 3 Frequency distributions of grain length, grain width and length-width ratio in the BC_4F_2 population containing 214 individuals in E9. **a** Grain length of paddy rice; **b** grain length of brown rice; **c** grain width of paddy rice; **d** grain width of brown rice; **e** length-width ratio of paddy rice; **f** length-width ratio of brown rice

Interestingly, the 50 large-LWR individuals all showed long rice grains, and the 62 non-segregating small-LWR lines all had short rice grains without segregation. Additionally, highly significant positive correlations, $r = 0.912^{***}$ and 0.908^{***}, were observed between the grain length and the LWR of paddy rice and brown rice, respectively. These results suggested the large LWR was also controlled by the *gl-3* gene in the BC_4F_2 population. Though the LWR was a complex trait of grain length and grain width, QTLs for grain width had relatively small effects on the phenotypic variaton of the LWR in the CSSL18/Asominori F_2 population. The Fig. 3c,d

clearly indicated minor factors conferred the phenotypic difference of grain width between CSSL18 and Asominori. Therefore, only the phenotypic data of grain length would be used for the subsequent primary and fine mapping of the *gl-3* gene.

Small-scale mapping of the *gl-3* gene

By means of linkage analysis using the genotype data of both the *gl-3* gene and SSR and EST markers previously published, we detected seven markers linked to the *gl-3* gene on the long arm of chromosome 3 (Fig. 4a). However, no molecular markers were found on the other side of the *gl-3* gene, possibly because there are relatively few markers developed in the centromeric region of chromosome 3 (Harushima et al. 1998; McCouch et al. 2002; Wu et al. 2002). Therefore, DNA sequences of two BAC/PAC contigs (OSJNBb004M10 and OSJNBb0087G11) of Nipponbare were used to develop five polymorphic SSR markers (Table 2). Subsequently, the *gl-3* gene was mapped to a genomic region of 5.7 cM in length, at 1.8 cM from RMw312 and 3.9 cM from RM7370 (Fig. 4a).

High-resolution mapping of the *gl-3* gene

For high-resolution mapping, 499 BC_4F_2 plants of long-grain homozygotes were analyzed for the recombination frequency between the *gl-3* locus and newly developed SSR markers in the genomic region RMw312-RM7370 (Fig. 4a). Eleven polymorphic SSR markers were designed from seven BAC/PAC contigs of Nipponbare (Fig. 4b; Table 2) and used for linkage analysis. Of these, RMw357 and RMw353 both had three recombinants with the *gl-3* locus in the 499 homozygous plants (Fig. 4c). Thus, the *gl-3* gene was precisely located between RMw357 and RMw353 within an interval of 0.6 cM genetic distance (Fig. 4c). Since RMw357 and RMw353 were produced from sequence information of the contigs OSJNBb0074M06 and OSJNBa0002D18, respectively, the sequences of the two contiguous contigs were analyzed with the Sequencer Program (Gene Code Corporation, Mich., USA) to further confirm the overlapping sequences and the physical distance between the *gl-3* gene and these closely linked markers. Consequently, the genomic region containing the *gl-3* locus was narrowed down to an 87.5-kb fragment defined by RMw357 at 16469.4 kb and RMw353 at 16556.9 kb on chromosome 3 (http://www.gramene.org) (Fig. 4c).

Physical-to-genetic distance ratio near the centromere of chromosome 3

By comparing the position of the centromere of rice chromsome 3 as defined by molecular markers (Singh et al. 1996; Harushima et al. 1998; Chen et al. 2002), we deduced that QTL *qGL-3a*, which was detected in the

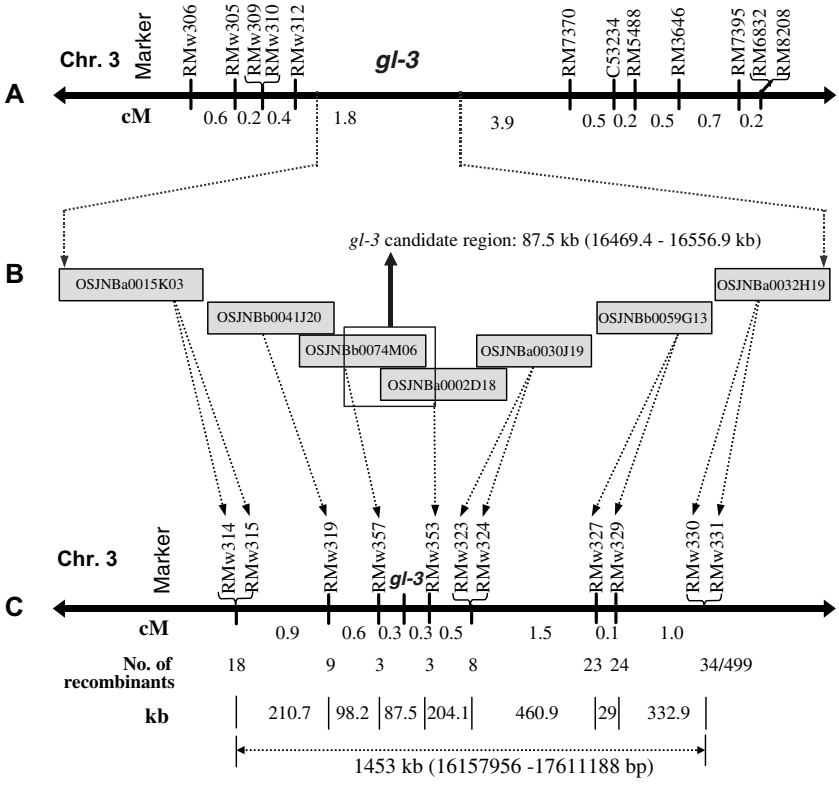


Fig. 4 Genetic and physical maps of the *gl-3* locus on rice chromosome 3. **a** Primary mapping of the *gl-3* gene; **b** BAC/PAC contigs around the *gl-3* locus; **c** Fine mapping of the *gl-3* gene

interval C80-C1677, lay in the genetically bounded centromere, with C1677 on the short arm and C80 on the long arm. The 5.2-cM long high-resolution linkage map containing the *gl-3* locus could be divided into seven intervals, namely, RMw315-RMw319 (0.9 cM), RMw319- RMw357 (0.6 cM), RMw357-RMw353 (0.6 cM), RMw353-RMw323 (0.5 cM), RMw323-RMw327 (1.5 cM), RMw327-RMw329 (0.1 cM), and RMw329-RMw330 (1.0 cM) (Fig. 4c). The corresponding physical distances were 210.7, 98.2, 87.5, 204.1, 460.9, 29.0 and 332.9 kb (Fig. 4c), giving physical-to-genetic distance ratios of 234.1, 163.7, 145.8, 408.2, 307.3, 290.0 and 332.9 kb/cM, respectively. Thus, the average ratio was 279.4 kb/cM in the 1,453-kb region between RMw315 and RMw330, which is consistent with the genome-wide average physical-to-genetic distance 244 kb/cM (Chen et al. 2002).

Discussion

Fine mapping of QTLs underlying rice quality as Mendelian factors

In this study, we detected and characterized six QTLs controlling rice grain length, by using the RIL population in four environments (Table 3). CSSLs harboring individual QTLs (QTL–CSSLs) were used to evaluate gene action and expression stability of each detected QTL in eight environments (Fig. 2). One stable major QTL, *qGL-3a*, was separated as a single recessive gene *gl-3* by the test of advanced backcross progenies (Fig. 3a, b), and was narrowed down to an 87.5-kb genomic region by using a larger secondary segregating population (Fig. 4c). Based on the available sequence annotation (http://www.rgp.dna.affrc.go.jp; http://www.softberry.com; http://www.ncbi.nlm.nih.gov/BLAST), there were 12 predicted candidate genes in the 87.5-kb target region. Among these genes, seven had unknown functions, and the corresponding proteins of five genes with functional annotation included (1) a putative polyprotein; (2) a putative retrotransposon GAG protein; (3) a putative serine/threonine protein kinase; (4) a putative GAG-POL precursor; and (5) a putative transposase. However, it is difficult to determine which candidate gene should be the *gl-3* gene, due to shortage of understanding the formation mechanism of grain length and co-segregating markers with the *gl-3* gene. In order to clone the *gl-3* gene, sequence comparison of the 12 candidate genes is been conducting between Asominori and CSSL18. Meanwhile, high-resolution mapping is ongoing by using 10, 560 BC_4F_2 plants and cleaved amplified polymorphic sequence (CAPS) and single nucleotide polymorphism (SNP) markers. These results clearly indicate that the major QTL can be treated as a single Mendelian factor for further research on functional genomics of quantitative traits.

Interestingly, the 87.5-kb genomic region containing the *gl-3* gene overlapped partly the 93.8-kb interval harboring the *gw*3.1 gene underlying rice grain weight (Li et al. 2004a). Otherwise, the conclusion of the dominant *O. rufipogon* allele *Gw*3.1 conferring small seed size is consistent with that of the dominant Asominori allele *Gl*3.1 controlling short grain and small LWR in this study. So we can't exclude the possibility that the *gl-3* gene is the same as the *gw*3.1 gene. It is a good chance to elucidate the relationships of development and domestication among grain weight, grain length and LWR by using different germplasm in different laboratories.

So far, many major QTLs have been precisely mapped and cloned in previous reports, for instance, heading data and seed dormancy in rice (Yano et al. 2000; Takahashi et al. 2001; Takeuchi et al. 2003), fruit weight and shape in tomoto (Frary et al. 2000; Liu et al. 2002), and vernalization in wheat (Yan et al. 2003, 2004). However, there have been few examples, to our knowledge, in rice quality. Several studies in our laboratory have demonstrated that utilization of QTL–CSSLs and their derivative segregating populations is a powerful strategy for estimating gene action of QTLs and to conduct fine mapping of QTLs underying complex rice appearcence and cooking quality traits, such as endosperm chalkiness (Wan et al. 2004b), grain width, breakdown viscosity and setback viscosity of endosperm starch (unpublished data).

Implications for marker-aided QTLs pyramiding

One of the most important objectives of QTL mapping is to apply MAS for genetic improvement of quantitative traits. Most successful research on MAS application has been conducted for resistances to diseases and insects conferred by major genes (Mackill and Ni 2001), and few efforts on MAS for QTLs have been reported in rice quality improvement, possibly bacause information on QTL epistasis, QEI effects, gene action of QTL in the new genetic background, and DNA markers closely linked to target QTLs is particularly lacking (Li 2001). Otherwise, rice grain length is highly heritable and is easy to score, whereas the continuous phenotypic segregation in progeny and trait measurement after the reproductive stage make it difficult for breeders to improve grain appearance efficiently using conventional selection methods. Thus, rice grain length is a good candidate trait for MAS experiments.

For these reasons, the IR24 allele at *qGL-3a* may be recommended as a desirable target gene/QTL for improving rice appearance quality by using a MAS strategy. This point can be demonstrated from consideration of the following seven aspects. (1) Gene action of *qGL-3a* was significant and stable for increasing rice grain length not only in the isogenic background of Asominori but also in the recombinant background of Asominori and IR24 (Table 3, Fig. 2); (2) Expression

stability of *qGL-3a* was high across multiple environments, that is, QEI did not influence gene action of *qGL-3a* (Table 3; Wan et al. 2005); (3) *qGL-3a* accounted for a large proportion of the overall phenotypic variation, with average PVEs of 32.8% and 34.6% in the CSSL and RIL populations, respectively (Table 3; Wan et al. 2005); (4) By reference to the high-density linkage maps (Harushima et al. 1998; McCouch et al. 2002), *qGL-3a* may be seen to occur at the same locus as QTLs reported previously in the centromeric region of rice chromosome 3 (Huang et al. 1997; Redona and Mackill 1998; Tan et al. 2000; Li et al. 2004b; Aluko et al. 2004), indicating that *qGL-3a* could express in different genetic populations or germplasm; (5) epistatic interaction between *qGL-3a* and a modifying factor in the interval C3029C-C2340 could promote gene expression of *qGL-3a* in a complex background (Table 4); (6) mapping *qGL-3a* as a single Mendelian factor, *gl-3*, would have a great impact on MAS breeding, since the deleterious alleles near the *gl-3* locus could be removed easily by MAS; (7) seven flanking SSR markers, RMw314, RMw315, RMw319, RMw357, RMw353, RMw323 and RMw324, could readily be used by rice breeders for marker-assisted introgression of the *gl-3* gene into a leading cultivar, because the interval of 2.6 cM long (Fig. 4c) is small enough to reduce linkage drag (Frisch et al. 1999). Therefore, the *gl-3* gene, the seven SSR markers and the four *gl-3*-CSSLs (Fig. 2) should be valuable for MAS improvement in rice quality breeding.

Moreover, since several QTLs for grain width, endosperm chalkiness and starch viscosity have been dissected into single Mendelian factors and precisely mapped by using QTL-CSSLs and their derivation progenies in our laboratory, QTL pyramiding by MAS should be feasible, that is, simultaneous transfer of a large number of desirable QTLs for target traits using the tightly linked DNA markers and target CSSLs. The corresponding work is ongoing.

Genetic complexity due to minor QTLs and epistatic interactions

Although progress has been made in fine mapping and map-based cloning of major QTLs, experimental constraints have limited our knowledge of minor QTLs, which could be responsible for a large proportion of trait variation. In this study, five minor QTLs were detected in the RIL population (Table 3). Their gene action was significantly environment-specific and in some instances the gene effect even had an opposite direction in QTL-CSSLs with the genetic background of Asominori (Fig. 2). Considering the effects of environment and genetic background on QTL expression, one explanation might be epistatic interactions between alleles at a main-effect QTL and a background locus, occurring more frequently in RIL populations (Li 2001). Table 4 showed that three QTLs (*qGL-2*, *qGL-5* and *qGL-9*) interacted with modifying factors in the intervals XNpb177-C600, C718B-C104A and C1003A-C1172, with PVEs of 5.90, 6.21 and 4.45%, respectively. However, these epistatic effects might not exist in the isogenic background of Asominori. Similarly, both Li et al. (2003) and Kroymann and Mitchell-Olds (2005) found that epistasis played an important role in affecting the gene action and expression stability of QTLs, especially for minor QTLs. For *qGL-7*, the target CSSL46 showed higher phenotypic values than those of Asominori across all the eight environments, but the effects obviously resulted from the IR24 allele at *qGL-3a* since CSSL46 carried both *qGL-3a* and *qGL-7*. These results imply that it will be relatively difficult to precisely map and clone QTLs *qGL-2*, *qGL-5*, *qGL-7* and *qGL-9*.

Apart from QTL *qGL-3* located at the same location as *qGL-3a* detected in this study, three other minor QTLs for grain length (*qGL-1*, *qGL-2* and *qGL-4*) were detected in eight, three and five of the eight environments, respectively, by using a population of 66 CSSLs (Wan et al. 2005). The three QTLs were different from any QTL identified in this study. Moreover, the gene action of *qGL-1* was significant and stable in the background of Asominori across multiple environments. High-resolution mapping of *qGL-1* is in progress for future map-based cloning. Interestingly, a similar phenomenon occurred in *Hd-6*, which was not detected in the F_2 population but recognized in advanced backcross progeny, as reported by Yamamoto et al. (2000). Thus, CSSL mapping is typically more efficient than primary populations in fine mapping of individual QTLs, even minor QTLs.

Normal recombination frequency observed near the centromere

Since recombination has been severely suppressed around the centromere due to megabases of highly repetitive DNA (Chen et al. 2002; Cheng et al. 2002), a map-based cloning strategy will not be feasible for the isolation of genes in centromeric or pericentromeric regions. In this study, *qGL-3a* was located in the genetically bounded centromere of chromosome 3 (Fig. 4a). However, no evidence of recombination suppression was observed in the course of fine mapping the *gl-3* gene. This is strongly supported both by the highly informative recombinants in BC_4F_2 and BC_4F_3 populations and also by the relationship between physical distance and genetic distance around the *gl-3* gene (Fig. 4a–c). The same phenomenon appeared when high-resolution mapping was performed for the *gw*3.1 gene in the pericentromeric region of chromosome 3, by using a set of NILs derived from the cross of Jefferson × IRGC105491(Li et al. 2004a).

For the entire rice genome, the average physical distance per cM is 244 kb, but this ratio varies with position along the chromosome, with centromeric regions exhibiting ratios of >1 Mb/cM (Chen et al. 2002). In this study, the average ratio was 279.4 kb/cM in the

1,453-kb region between RMw315–RMw330 (Fig. 4c), which is consistent with the genome-wide average physical-to-genetic distance. But this is unexpected on the basis of reports of recombination suppression near the centromeres in interspecific crosses (Causse et al. 1994; Zeng et al. 2002; Liu et al. 2004). Therefore, elucidation of fine structure and in-depth characteristics around the centromere of rice chromosome 3 will be a challenge to molecular geneticists.

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